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PHYTOCHEMICAL ANALYSIS OF *ANDROGRAPHIS PANICULATA* EXTRACT AND ITS POTENTIAL AS ANTI BACTERIAL GROWTH AND ANTI BIOFILM FORMATION AGAINST *ESCHERICHIA COLI* AND *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Antibiotic resistance remains a significant challenge and a global threat in the treatment of infectious diseases. Gram-positive and Gram-negative bacteria exhibit tolerance towards antibacterial agents given their ability to synthesize biofilm. *Andrographis paniculata* is a well-known medicinal plant with various pharmacological properties. This study aimed to evaluate the antibacterial and antibiofilm activity of *A. paniculata* extract against *Escherichia coli* and *Staphylococcus aureus.* Various crude ethanol extract concentrations of *A. paniculata* were tested for their antibacterial potencies using the Kirby-Bauer disc-diffusion method for 24 h. Phytochemical analysis was confirmed using UV-visible spectroscopy and gas chromatography-mass spectrometry. Interactions between the bacterial cell surface and the extract were visualized using Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM). The results showed that the inhibition zones ranged from 2.17±0.77 to 10.59±0.26 mm and from 6.20±0.52 to 11.67±0.12 mm for *E. coli* and *S. aureus*, respectively. Moreover, all tested concentrations of *A. paniculata* were able to inhibit the formation of biofilm in these two strains. FTIR results indicated that the extract affected functional groups on the bacterial cell, while SEM micrographs confirmed that biofilm did not form when the bacteria were in contact with the extract. Furthermore, 57 metabolites in the extract were identified, of which palmitic acid and 9-octadecenal were the predominant phytochemical compounds. In conclusion, *A. paniculata* ethanol extract exhibits potential as an antibacterial agent due to its ability to control bacterial growth and biofilm formation. The higher the extract concentration used, the more potent its effect. These antibacterial activities were facilitated by the active phytochemical components.

Keywords: Antibacterial, antibiofilm, resistance, ethanol extract, phytochemical

INTRODUCTION

Microbial infection remains a global health concern due to the development of antimicrobial resistance (AMR) and tolerant strains. The misuse of antimicrobial drugs for treating infectious diseases has exacerbated the occurrence of AMR and the acquisition of tolerance, although spontaneous mutations and evolution of certain microbes may also play a significant role (**Aparicio-Blanco *et al*., 2024**). As a result, treating infectious diseases requires longer hospitalization and higher medical costs, leading to an increased likelihood of treatment failure and possibly death (**Matsunaga and Hayakawa, 2018**). It was reported in 2019 that AMR accounted for an estimated 1.27 million deaths worldwide and was associated with 4.95 million deaths annually (**Murray *et al*., 2022**).

Gram-positive and Gram-negative bacteria can synthesize biofilm (**Rewatkar, 2013**), which allow communities of microorganisms to attach to each other and/or onto biotic and abiotic surfaces. Although the biofilm allows bacteria to withstand harsh environmental conditions, i.e., changes in temperature, nutrient deprivation, and dehydration, it also provides increased resistance to antibiotics, allows evasion of detection from the immune system, and affords avoidance from other stressors (**Divakar *et al*., 2019**), leading to persistent and recalcitrant infections (**Idrees *et al*., 2021**). *E. coli* and *S. aureus* are common bacteria involved in the community and hospital-acquired bacterial infections (**Chouhan *et al*., 2017**) as well as infections from foodborne pathogens (**Bai*et al*., 2021.; Romli *et al*., 2023**). They are types of bacteria that produce biofilms to improve their survival (**Jang *et al*., 2017**). Thus, *E. coli* and *S. aureus* are predominant contributors to death and illness associated with antibiotic resistance. In 2019, *E. coli* ranked first as the pathogen associated with 829,000 deaths due to AMR, especially in cases of urinary tract infections and sepsis (**Daneman *et al*., 2023**). In the same year, *Methicillin-Resistant Staphylococcus aureus* (MRSA) was also reported as the leading pathogen-drug combination of AMR, accounting for 121,000 deaths worldwide (**Chew *et al*., 2023**).

The formation of biofilm plays an important role in antibiotic resistance since it renders standard treatments for bacterial infection including antibiotics, phage therapy, quorum sensing inhibitors, and monoclonal antibody therapy ineffective. As a result, finding an alternative treatment to alleviate multidrug resistance by preventing the formation of biofilms, has become a focus of interest in microbiological research. Novel antimicrobial candidates are being developed, and the bioactive compounds present in natural products, such as plant extracts are being explored.

Plant metabolites have played an important role in treating a wide range of microbial infections for several decades due to their low cytotoxicity. The use of herbal plants as antimicrobial agents is hypothesized to be effective and safe given it is relatively cheap and rarely causes side effects (**Majumdar *et al*., 2020**). *A. paniculata*, commonly known as the "King of Bitters", is a member of the *Acanthaceae* family and a well-known medicinal plant in several Asian countries. The active ingredients of *A. paniculata* are diterpenes, diterpenoids, glycosides, and flavonoids. Together, these metabolites are reported to have various biological activities such as antibacterial, antioxidant (**Muhamad Alojid *et al*., 2021**), anti-inflammatory, anti-atherosclerotic, and anti-platelet aggregation properties (**Hossain*et al*., 2021**). An ethanol-based crude extract of *A. paniculata* has been reported to inhibit the growth of *S. aureus* (**Sholihah *et al*., 2022**), as well as to prevent biofilm formation by *Pseudomonas aeruginosa* (**Majumdar *et al*., 2020**). AMR is predicted to increase if campaigns on promoting the rationale use of antibiotics failed, leading to higher morbidity, mortality, and healthcare costs. Therefore, exploring novel antimicrobial agents from natural sources is gaining increasing interest as an alternate antibiotic option. *A. paniculata* has previously been reported in several studies to exhibit antibacterial and antibiofilm activity. This study focused on exploring the active metabolites of *A. paniculata* crude ethanol extract and investigating the inhibitory effect of *A. paniculata* crude ethanol extract on the bacterial growth of *E. coli* and *S. aureus* and against biofilm formation by *E. coli* and *S. aureus*. Furthermore, how the *A. paniculata* extract interacted with the bacterial membrane was also examined to gain insight into the mechanism of *A. paniculata* prevented biofilm formation. This study provides significant results on the discovery of a natural antimicrobial agent, namely *A. paniculata*, which can prevent AMR against *E. coli* and *S. aureus* by inhibiting their growth and biofilm formation.

MATERIAL AND METHODS

Plant collection and extraction

A. paniculata was collected in Yogyakarta, Indonesia (7"43'48.7"S and 110°21'14.7"E). The air-dried plant leaves were extracted separately with 70% ethanol (500 ml/100 g) at room temperature. The final ethanol extract was filtered using filter paper (Whatman No. 1; GE Healthcare) and evaporated using a rotating vacuum evaporator at 40˚C (Buchi R-114; Buchi Labortechnik AG).

Bacterial strains and cultivation

The strains of *E. coli* (ATCC 32518) and *S. aureus* (ATCC 25923) used in the present study were deposited in the Laboratory of Microbiology, Universitas Negeri Yogyakarta. The bacteria were routinely grown in Nutrient Agar (NA, Oxoid) at 37˚C. Bacteria were cultivated aerobically in Nutrient Broth (NB, Oxoid) for 24 h prior to inoculum production.

Antibacterial activity assessment

To evaluate the antibacterial activity of the crude extract, the Kirby-Bauer disc infusion assay was employed following a protocol adapted from (**Zouaghi *et al*., 2021**). The standardized bacterial broth culture was streaked evenly on sterile Muller-Hinton agar plates. Sterile paper discs (Oxoid) saturated with 100 µl of each extract at various concentrations (0, 0.156, 3.125, 6.25, 12.50, 25.00, 50.00, and 75.00 mg/ml) were placed on the plates and incubated for 24 h at 37˚C. For comparison, chloramphenicol (0.2 mg/ml) and 1% DMSO (MilliporeSigma) were used as a positive and negative control, respectively. Three replicates were made for each of the bacterial isolates. Finally, the plates were incubated at 37˚C and the antibacterial activity was measured by calculating the inhibition zone diameter around the paper discs every 3 h for 24 h. The calculated values are presented as the mean ± SD. The Minimum Inhibitory Concentration (MIC) was defined as the lowest sample concentration that completely inhibited the growth of the bacteria. The MICs of the plant extract in a series of concentrations were tested in NB for 24 h at 37˚C. Briefly, cultures with an OD_{600} of 0.8, were inoculated into media solutions for each treatment in triplicate. Bacterial cultures were incubated at 37˚C for 24 h, with agitation at 100 r/min.

Antibiofilm evaluation

The modified biofilm formation technique (**Giovanella *et al*., 2017**) was used to assess the effect of various extract concentrations of *A. paniculata* on biofilm formation by *E. coli* and *S. aureus*. Without agitation, the bacteria were cultured in 200 µl NB, together with the varying extract concentrations of *A. paniculata* at 37˚C and pH 7.4. After 48 and 96 h of incubation, the culture media was aspirated and rinsed three times with 250 µl 0.9% NaCl solution, then dried at 40˚C for 40 min. Adherent cells were stained with 1% gentian violet for 10 min before the excess dye was removed. Before washing with 95% ethanol, the biofilm was rinsed five times in a 0.9% NaCl solution. Biofilm absorbance was determined using an ultraviolet-visible spectrophotometer Genesys 10S (Thermo Fisher Scientific, Inc.) at 595 nm wavelength.

Phytochemical analysis

The samples were analyzed at the Integrated Research and Testing Laboratory Universitas Gadjah Mada to quantify the content of phenols (**Kupina *et al*., 2019**), flavonoids (**Nurcholis *et al*., 2021**), alkaloids **(Fazel *et al*., 2010**), tannins (**Amorim *et al*., 2008**), and saponins (**Vador, *et al*., 2012**). Gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed using a QP2010M mass spectrometer (Shimadzu) at the Organic Chemistry Laboratory of Universitas Gadjah Mada, following the previous protocol as described in **Rakhmawati (2022).** The chemical components were identified by mass spectral analysis of the GC peaks using the National Institute of Standards and Technology and Wiley databases (**Abid *et al.*, 2017**).

Visualization of the interactions between active metabolites of the extract and the bacteria

Fourier Transform Infrared Spectroscopy (FTIR) was performed on *E. coli* and *S. aureus* cells before and after treatment with 12.5% plant ethanol extract to determine the identity of surface molecules and the chemical structure of the bacteria. The samples were mechanically lysed in an agate mortar before being conditioned in Potassium Bromide pallets and tested using 8201PC (Shimadzu). For Scanning Electron Microscopy (SEM) analysis, circular Polyethylene (PE) films (50 mm in diameter) were soaked in the NB broth with or without the 12.5% *A. paniculata* ethanol extract supplementation. The media was then inoculated with *E. coli*, and cultivated without agitation for 96 h. The PE films were removed from the liquid medium and washed with distilled water carefully to liberate the loosely attached bacteria. Next, the samples were placed into pellets with a carbon-coated copper grid for SEM-EDX analysis (JSM-6510LA, JEOL Ltd).

Statistical analysis

The antibacterial and antibiofilm activity was statistically analyzed using SPSS version 25 (IBM Corp.). A one-way ANOVA followed by a post-hoc Tukey test was used to compare the differences between multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS AND DISCUSSION

Result

The potency of *A. paniculata* extract as an effective antibacterial agent to manage multidrug resistance was tested by looking at their effect on the inhibition zone, MIC, and prevention of biofilm formation against *E. coli* (Gram-negative), and *S. aureus* (Gram-positive) bacteria. The diameters of the inhibition zones are shown in Tables 1 and 2 and representative images of the MIC are shown in Figure 1. Furthermore, to justify the effect of the active metabolites of the plant extract on the bacterial cells, FTIR and SEM were used for better visualization, as shown in Figures 2 and 3, respectively. Inhibition of biofilm formation by both bacteria is presented in Table 3. In addition, the results of the phytochemical analysis of *A. paniculata* crude ethanol extract are shown in Table 4, and representative chromatograms are shown in Figure 4.

Table 1 Inhibition zone diameter (mm) of *A. paniculata* extract against *E. coli*

Treatment (mg/mL)	Incubation Time (h)							
	3	6	9	12	15	18	21	24
Control (-)	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
1.5623	7.38 ± 0.18^{b}	7.55 ± 0.28^{b}	7.12 ± 0.12^{b}	2.27 ± 3.94^{ab}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
3.125	7.96 ± 0.63^{bc}	8.04 ± 0.62^{b}	7.15 ± 0.35^{b}	4.66 ± 4.04^{ab}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
6.25	9.28 ± 0.75^{bc}	13.14 ± 6.72^{bc}	7.45 ± 0.49^{b}	6.27 ± 0.05^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
12.5	9.72 ± 0.83^{c}	10.02 ± 0.55^{b}	7.50 ± 0.08^{b}	6.27 ± 0.01^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
25	9.92 ± 0.86^{c}	10.56 ± 0.53^{bc}	7.51 ± 0.51^{b}	6.32 ± 0.06^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
50	9.95 ± 1.19^{c}	10.57 ± 1.68^{bc}	7.61 ± 0.63^{b}	6.35 ± 0.13^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
75	9.99 ± 0.75^{c}	10.59 ± 1.26^{bc}	8.30 ± 1.62^{b}	7.01 ± 0.94^{b}	2.17 ± 3.77^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Control (+)	12.22 ± 0.43^{d}	18.58 ± 1.14^{d}	18.95 ± 1.07^{c}	17.62 ± 1.13^{c}	16.35 ± 0.87^{b}	15.71 ± 0.62^{b}	14.00 ± 0.35^{b}	13.28 ± 0.48^{b}

Inhibition zone diameter is measured for each treatment group during incubation time from 3h to 24 h (Control (-): DMSO 1%; Control (+): Chloramphenicol 200 µg/mL). Values are presented as mean ± SD. Significant differences among various concentrations at each incubation time are indicated by different superscripted lowercase letters on the same column ($p < 0.05$, Anova test followed by Tukey, n = 27).

Table 2 Inhibition zone diameter (mm) of *A. paniculata* extract against *S. aureus*

Treatment (mg/mL)	Incubation Time (h)							
	3	6	9	12	15	18	21	24
Control (-)	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
1.5623	8.41 ± 0.87^{a}	8.52 ± 0.87^{b}	7.04 ± 0.38^{b}	6.47 ± 0.32^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
3.125	9.27 ± 0.32^{a}	9.41 ± 0.28^{bc}	7.06 ± 0.29^{b}	4.34 ± 3.77^{ab}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
6.25	10.36 ± 0.92^{a}	10.58 ± 0.99^{bc}	7.13 ± 0.38^{b}	2.06 ± 3.57^{ab}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
12.5	10.38 ± 0.26^{a}	10.81 ± 0.80^{bc}	7.22 ± 0.22^{b}	2.07 ± 3.59^{ab}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
25	10.68 ± 0.45^{a}	10.82 ± 0.47^{bc}	7.61 ± 0.23^{b}	6.78 ± 0.41^{b}	4.27 ± 3.70^{ab}	4.16 ± 3.60^{ab}	4.13 ± 3.57^{ab}	0.00 ± 0.00^{a}
50	40.20 ± 50.32^{a}	10.90 ± 0.65^{bc}	7.63 ± 0.86^{b}	6.96 ± 0.77^{b}	2.30 ± 3.98^{ab}	2.16 ± 3.75^{ab}	2.15 ± 3.73^{ab}	0.00 ± 0.00^{a}
75	11.58 ± 2.15^{a}	11.67 ± 2.12^{c}	7.93 ± 0.73^{b}	7.50 ± 0.69^{b}	7.16 ± 0.07^{b}	6.40 ± 0.10^{b}	6.30 ± 0.10^{b}	2.03 ± 3.52^{a}
Control (+)	57.50 ± 42.45^{a}	18.10 ± 0.27^{d}	21.57± 1.82^{c}	19.33 ± 1.01^{c}	17.64 ± 1.81^{c}	16.03 ± 1.80^{c}	15.41± 1.60^{c}	13.28 ± 2.05^{b}

Inhibition zone diameter is measured for each treatment group during incubation time from 3h to 24 h (Control (-): DMSO 1%; Control (+): Chloramphenicol 200 µg/mL). Values are presented as mean ± SD. Significant differences among various concentrations at each incubation time are indicated by different superscripted lowercase letters on the same column ($p < 0.05$, Anova test followed by Tukey, n = 27).

Antibacterial activity *of A. paniculata* extract at the MIC level. The results revealed that the different extract concentrations of *A. paniculata* exhibit different degrees of antibacterial activity against two tested isolates. The diameter of the growth inhibitory halos varied from 2.17±0.77 to 10.59±0.26 mm and from 6.20±0.52 to 11.67±0.12 mm for *E. coli* and *S. aureus*, respectively. The MIC of the different extract concentrations on the test organisms is shown in Figure 1.

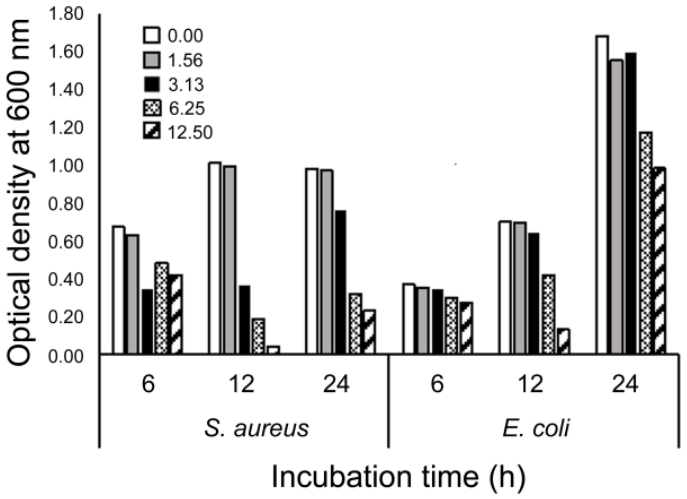

Figure 1 The MIC values of *A. paniculata* extract against *E. coli* and *S. aureus*
Antibiofilm activity of *A. paniculata extract.* There was no bacterial growth observed when treated with ≥25 mg/ml extract, therefore antibiofilm activity was only assessed at concentrations from 0.00 up until 12.50 mg/ml. The results of antibiofilm activity in the treated *E. coli* and *S. aureus* are shown in Table 3. All tested concentrations of *A. paniculata* inhibited the formation of biofilm by the bacteria. Even when treated with 12.50 mg/ml, the extract demonstrated antibiofilm activity, especially when incubated for 96 h.

Interaction between *A. paniculata* extract and the bacteria. FTIR analysis was performed on the bacterial cells to provide qualitative information regarding the main functional groups present on the cell wall that may be affected by the plant extract. Comparisons of bacterial cell spectra before and after contact with the extract are shown in Figure 2 and Table 4.. The results did not show systematic shifts of the bands after the addition of the plant extract, but punctual and discrete shifts of certain bands. Meanwhile, SEM was used to observe the phases of biofilm formation, bacterial interactions, and extracellular polymer production. The representative results of this analysis on *E. coli* are shown in Figure 3.

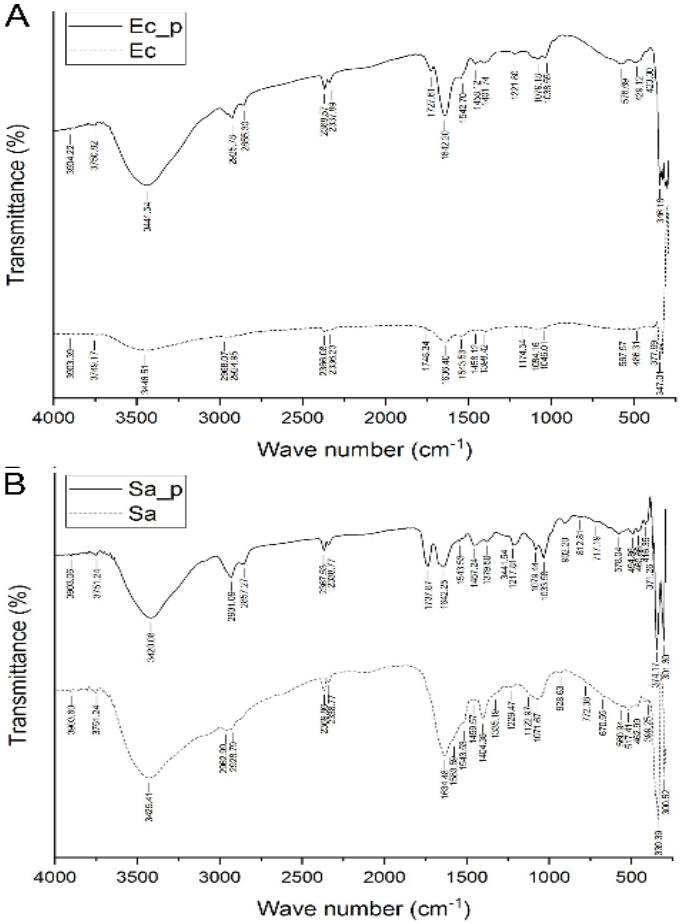

Figure 2 FTIR Spectra of *E. coli* (A) and *S. aureus* (B) for 24h showing two types of line, solid line (top) and dotted line (bottom). The top line indicates the FITR spectra of bacterial biomass added with the extract, while the bottom line refers to the one without the extract. Transmission peaks showed by the the top line are sharper than the bottom line. Adding extracts causes a shift in peaks and the emergence of new peaks, indicating the presence of bond stretching due to the interaction between the extract with the major functional groups of the bacterial cells (see also Table 4).

Table 3 The effect of *A. paniculata* extract on the biofilms of *E. coli* and *S. aureus*

Extract concentration (mg/mL)	Incubation time (h) for *E. coli*		Incubation time (h) for *S. aureus*	
	48	96	48	96
0	0.075 ± 0.001^{e}	0.097 ± 0.002^{e}	0.095 ± 0.001^{e}	0.103 ± 0.002^{e}
1.56	0.063 ± 0.002^{d}	0.080 ± 0.001^{d}	0.084 ± 0.002^{d}	0.092 ± 0.001^{d}
3.125	0.053 ± 0.001^{c}	0.069 ± 0.001^{c}	0.076 ± 0.003^{c}	0.083 ± 0.002^{c}
6.25	0.046 ± 0.001^{b}	0.055 ± 0.001^{b}	0.062 ± 0.001^{b}	0.072 ± 0.001^{b}
12.50	0.036 ± 0.001^{a}	0.043 ± 0.001^{a}	0.053 ± 0.002^{a}	0.065 ± 0.001^{a}

Values are presented as mean ± SD. Significant differences among various concentrations at each incubation time are indicated by different superscripted lowercase letters on the same column ($p < 0.05$, Anova test followed by Tukey, n = 15)

Table 4 FTIR transmittance peaks and corresponding functional group assignments

E.coli				*S. aureus*			
Without plant extract		**With plant extract**		**Without plant extract**		**With plant extract**	
Wave number (cm^{-1})	**Functional group**	**Wave number (cm^{-1})**	**Functional group**	**Wave number (cm^{-1})**	**Functional group**	**Wave number (cm^{-1})**	**Functional group**
3446.51	O-H;N-H	3441.54	O-H;N-H	3429.41	O-H;N-H	3420.08	O-H;N-H
2366.06	C-H	2925.78	C-H	2962.99	C-H	2931.09	C-H
1636.40	C=C	2855.30	C-H	2928.79	C-H	2857.27	C-H
1543.53	C=C	2368.57	C-H	2369.86	C-H	2367.27	C-H
1458.12	C-N; C-H	2337.89	C-H	1634.48	C=C	1737.87	C=C
1398.42	C-H	1642.20	C=C	1404.38	–COO	1642.25	C=C
		1542.70	C=C	1071.67	P=O	1457.24	C-N; C-H
		1458.12	C-N; C-H			1217.81	C-N; C-O
		1401.74	C-O; C-N; C-H			1033.58	P=O; P-O-H
		1079.18	P=O				
		1038.55	P=O; P-O-H				

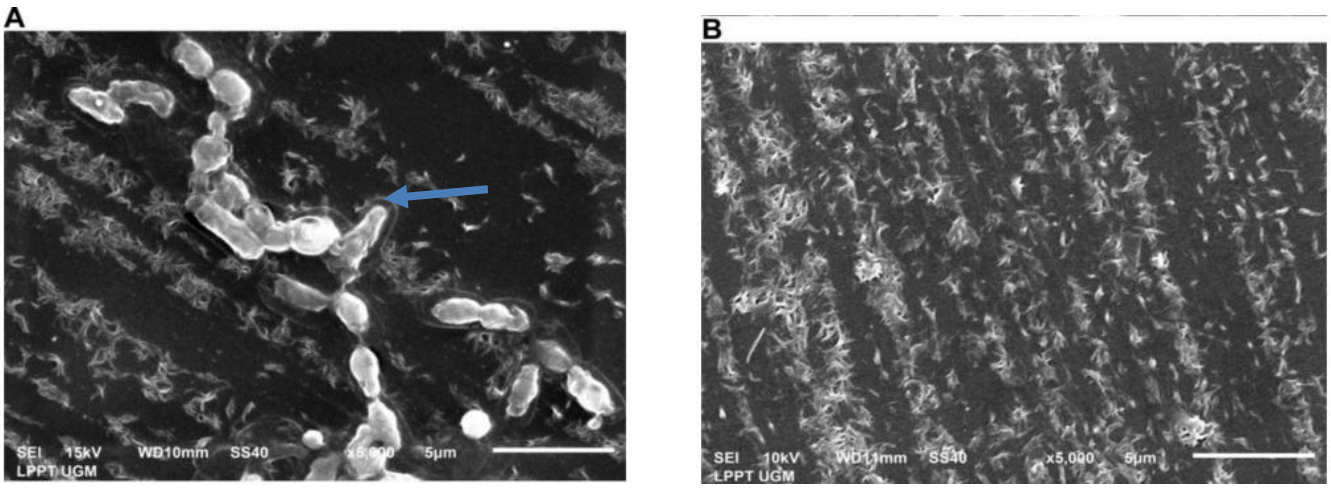

Figure 3 SEM micrographs of *E. coli* colonization on plastic surfaces, showing the surfaces without (A) and with the addition of plant extract (B). Note: blue arrow indicates bacterial cells attached to the surface of the plastic (A), but no such phenomenon could be found in B, meaning that there are no bacterial cells attached to plastic's surface.

Table 5 Phytochemical components identified in the crude extract ethanol of *A. paniculata*

No	RT	Identified components	Molecular weight	Peak (%)
20	36.534	Palmitic acid	256	21.15
28	40.052	9-Octadecenal	266	15.26
26	39.480	Palmitaldehyde	398	8.23
50	51.310	Trans-Farnesol	222	5.64
25	39.229	11-Octadecenoic acid	296	4.98

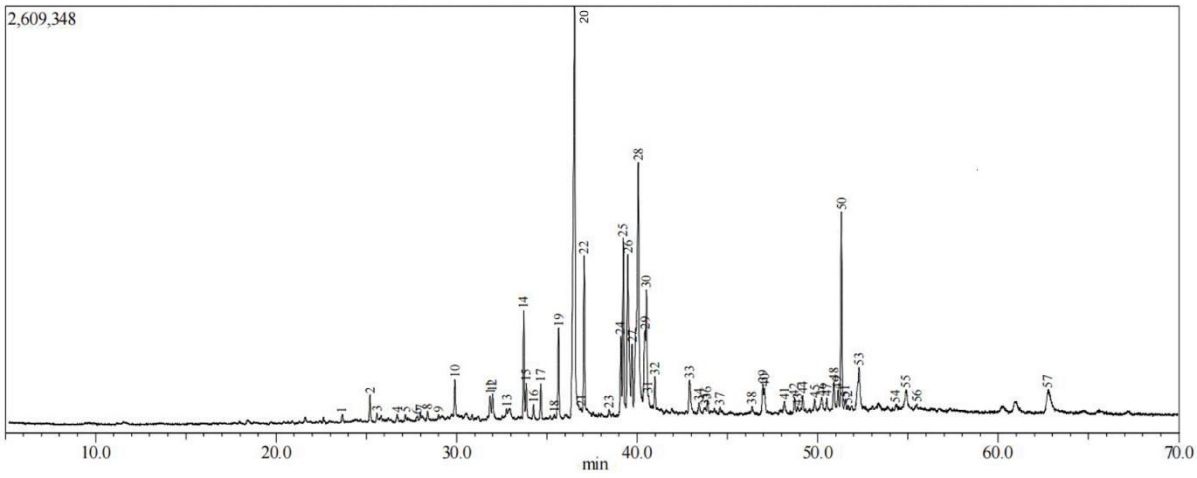

Figure 4 Chromatogram of the crude extract ethanol of *A. paniculata*

Phytochemical analysis. GC-MS analysis was used to provide detailed qualitative and quantitative information regarding the composition of the crude extract. The GC-MS analyses revealed 57 compounds, with the most abundant being palmitic acid, 9-octadecenal, palmitaldehyde, trans-farnesol, and 11-octadecenoic acid (Table 5 and Figure 4).

DISCUSSION

E. coli and *S. aureus* bacteria are the predominant pathogens associated with high morbidity and mortality rates due to AMR. Promoting the rational use of antibiotics remains a valuable approach used globally to prevent AMR. Another strategy to overcome this issue is to explore the potency of extracts from natural sources as alternative antimicrobial agents. In the present study, the potency of *A. paniculata* extract as an antibacterial candidate was assessed by determining its effects on the growth and biofilm formation of *E. coli* and *S. aureus*. The use of crude extracts showed a more concentrated solution allowed for the holistic targeting of numerous checkpoints of microbial metabolism. The use of natural resources is gaining increasing popularity due to their ease of usage and safety (**Tanwar *et al*., 2016**). The findings of the present study demonstrated that *A. paniculata* extract was capable of inhibiting the growth of both *E. coli* and *S. aureus*, with the optimal effect observed after 6 h of incubation time. In addition, the plant extract was also able to prevent biofilm formation of both bacteria, particularly during a 96-h incubation period.

We found the results of the inhibition zone values were overall 2-11 mm, which is classed in the weak and moderate response category and lower than another study use *A. paniculata*'s extract, which demonstrated the potency of bacterial growth inhibition around 10-16 mm (**Geetha and Alexander, 2017**). In addition, it was observed that a higher concentration of the *A. paniculata* extract resulted in more potent inhibition, and thus higher antibacterial activity. The ability to exhibit antibacterial activity depends on several factors, including the various components that make up the extract, inoculum volume, bacterial growth phase, culture medium, pH, exposure duration, temperature, and the presence of other antimicrobials (**Arini *et al.*, 2020**). Thus, future studies with higher extract concentrations may be useful to evaluate the actual antibacterial properties of *A. paniculata* extracts.

In the present study, it was found that MIC values of 5-15 mg/ml were considered to have moderate antimicrobial activity (**Monteiro *et al*., 2020**). This finding is higher than a previous study, which reported the MIC of *Andrographolide sulfonate* for MRSA was 50 µg/ml (**Zhang *et al*., 2021**). As mentioned above, the antibacterial activity was found to be optimal for up to 9 h of incubation time. Based on the OD value measured after 6 and 12 h of incubation time, the growth of *E. coli* at concentrations <12.5 mg/ml was higher than that of *S. aureus*. Furthermore, although the antibacterial activities for both tested isolates were optimal for 6 h and decreased gradually after 9 h of incubation time, the growth inhibition was longer for *S. aureus* (up to 24 h) than for *E. coli* (up to 15 h). All in all, we observe that the antibacterial potency appears more sensitive towards *S. aureus* (Gram-positive bacteria) than *E. coli* (Gram-negative bacteria), or on the other hand, the antibacterial action of this *A. paniculata* extract is presumably less sensitive towards *E. coli* (Gram-negative) than *S. aureus* (Gram-positive) bacteria. However, this assumption needs to be treated cautiously since more experimental studies are needed to test the antibacterial potency of this extract against other Gram-positive and Gram-negative bacteria to generalize this important finding. Nonetheless, our results are in agreement with the findings of the results of studies using extracts from other plants (**Purba *et al.,* 2020**).

All tested concentrations of *A. paniculata* were able to inhibit the formation of biofilms in both bacteria. Even when using 12.5 mg/ml, the extract still demonstrated antibiofilm activity during the 96-h incubation period. Moreover, the higher the extract concentration was, the stronger the inhibition of biofilm formation was. Compared to other studies on biofilm formation by *E. coli* and *S. aureus*, our present study showed lower antibiofilm activity (**Rasool *et al*., 2018**). Another previous study on the effect of *A. paniculata* on bacterial growth and biofilm formation demonstrated that 250 µg/ml of the plant ethanol crude extract could inhibit the growth and biofilm formation of *Klebsiella pneumoniae* compared to the antibiotics (**Sah *et al.,* 2020**). Several factors can affect biofilm formation, namely genetic and environmental factors. The physiological heterogeneity of biofilms may include chemical gradients, adaptation to restricted environmental settings, and various genotypes and phenotypes that express different metabolic pathways within the population. The medium used to produce biofilms also has a significant effect on the level of biofilm formation (**Triveni *et al*., 2018**). The use of any substrates depends on the ability of a particular microorganism to adhere, and this is governed by several physical factors that help bacteria attach to solid substrates, substrate properties, and microbial properties (**Gupta and Devi, 2020**). The composition of media, pH, and temperature are also hypothesized to contribute to biofilm cohesion (**Hussein and Alsharifi, 2021**).

Supporting the results on the *in vitro* antibacterial effects of *A. paniculata* extract on *E. coli* and *S. aureus*, the FTIR spectrum in the present study showed no absorption peaks and the presence of new peaks, meaning that the results of transmission peaks in FTIR of *E.coli* and *S. aureus* cell biomass added with plant extracts are sharper than the control (without plant extracts). The treatment of adding extracts caused a shift in peaks and the emergence of new peaks. When those peaks occurred, it indicates the presence of bond stretching due to the interaction between the extract with the major functional groups (as seen in Table 4) of the bacterial cells. In Figure 2, we observed that although FTIR transmission peaks occur in both treated and untreated cell biomass of either E. coli and S. aureus, the cell biomass treated with plant extracts appear to be sharper than those without treatment (untreated). Moreover, when peak shifts and new peaks emerge, this indicates the occurrence of significant biochemical interactions between the plant extracts and the bacterial cells. Sharper peaks, shifts, and additional peaks in the spectrum show that the bacterial cells' primary functional groups are undergoing bond stretching and other vibrational alterations, as the direct results of interactions between bacterial macromolecules (proteins, lipids, nucleic acids, and polysaccharides) and the active ingredients in the plant extracts (such as phenolics, flavonoids, or essential oils) (**Kamnev *et al*., 2021**). These are due to the plant extract's effect on bacterial cells.

Furthermore, the potent antibiofilm activities of *A. paniculata* extract against *E. coli* and *S. aureus* are also supported by our findings using SEM on the observation of biofilm formation phases, bacterial interactions, and extracellular polymer production. This observation provides useful information on the shape, size, and positioning of individual bacteria inside the biofilms. Polyethylene plastic surfaces can act as a base for biofilm development since it has a rough and smooth top layer with low/high densities. The bacterial attachment to the surface can be promoted by the production of exopolymeric substances that promote bacterial adhesion on the plastic surface. However, bacterial cells were not observed on plastic surfaces when treated with the plant extract. This finding indicates that the biofilm did not appear because the bacterial cells had not attached directly to the plastic surface or interweaved their fibres. Surface conditioning and material hydrophilicity control biofilm attachment on plastics. It is known that bacterial cells colonize more quickly on hydrophilic surfaces (**Bhagwat, G *et al*., 2021**). By changing surface characteristics or directly preventing microbe adhesion, plant extracts interfere with this process and present opportunities for sustainable packaging and anti-biofilm coatings. Nonetheless, to maximize extract efficacy across various microbial communities, more investigation is required.

The antimicrobial and antibiofilm activities of *A. paniculata* extract are supported by its active phytochemical component. Using UV-visible spectroscopy and GC-MS analysis, it was found that the extract contained phenols (0.18% b/v); flavonoids (768.75 mg/l); tannins (0.42% b/v); alkaloids (205.27 mg/l); and saponins (0.37% b/v). Phenols can inhibit bacterial virulence factors including enzymes and toxins, interacting with the cytoplasmic membrane, and preventing the formation of biofilms (Miklasińska-Majdanik *et al*., 2018). Flavonoids can interact with cell membranes and thus affect the cell membrane's bioactivity, reducing the fluidity of the bacterial cell membrane, which is directly related to damage of cytoplasmic membranes or indirect damage through autolysis or weakening of the cell wall, and the subsequent osmotic lysis and influences the cell permeability (**Ampofo *et al*., 2020**), biofilm formation, porin expression, and interacts with several key enzymes (**Farhadi *et al*., 2019**). Saponins can increase membrane permeability resulting in the diffusion of the intercellular compound through the outer membrane and cell wall (**Setyani *et al*., 2020**), causing leakage of proteins and enzymes from the bacterial cell. Tannins can also inhibit biofilm formation and bacterial adhesion (**Girard and Bee, 2020**), as well as activate bacterial enzymes, and interfere with the course of proteins in the inner layers of cells, causing the bacterial cells to lyse (**Villanueva *et al.,* 2023**).

Furthermore, the results of GC-MS analyses identified 57 compounds, of which the five most abundant were palmitic acid, 9-octadecenal, palmitaldehyde, transfarnesol. and 11-octadecenoic acid. These findings may differ from previous studies due to differences in growing location, plant age, plant supplementation, as well as extraction solvent, and extraction procedure (Winitchai *et al*., 2021). Palmitic acid is well-established to exhibit antibacterial activity (**Awang-Jamil *et al*., 2021**). Octadecenal has an aldehyde functional group that inhibits pathogen bacteria such as *E. coli*, *Salmonella typhimurium* (**Patra, Das, and Baek 2015**), *Listeria innocua*, *S. aureus* (Hatami *et al.,* 2016). Palmitaldehyde also inhibits the growth of MRSA and *Cutibacterium acnes* (**Tsami *et al*., 2022**). Farnesol, known as a quorum-sensing molecule, affects bacterial cell membranes, leading to ionic imbalances, ion leakage, and ultimately, cell death. Additionally, this substance hinders the creation of biofilm by slowing the rate of glycan synthesis in the biofilm matrix (**Nowacka *et al*., 2020**). Finally, 11-Octadecenoic acid also possesses antibacterial activity (**Ghareeb *et al*., 2022**).

This study demonstrates significant findings on the antibacterial and antibiofilm potencies of metabolites from *A. paniculata* crude ethanol extract. However, it is of importance to note that our results are solely based on the plants cultivated and obtained from one geographical area that may limit justification whether *A. paniculata* from other cultivation area may provide similar results on their antibacterial and antibiofilm effects. Therefore, future studies utilizing *A. paniculata* or "bitter leaf plant" as antibacterial agent need to collect these plants cultivated from various different geographical sites, as well as to use and differentiate the leaves according to their different physiological state: young leaves vs old leaves. It is also important to apply the metabolome analysis if possible. Furthermore, since we tested the whole crude ethanol extract and did not isolate and tested specific metabolite, we emphasized that our significant findings could not be solely due to the effect of a specific compound, rather we highlight

that these results might due to the interaction of multi-compounds action (**Zouine *et al*., 2024**).

CONCLUSION

Various concentrations of crude ethanol extracts of *A. paniculata* (also known as Sambiloto) leaves demonstrate potent antibacterial and antibiofilm activities, with an optimal effect seen after 6 h of incubation time, in a concentration-dependent manner. These activities are likely attributed to the active phytochemical components. Between the two strains of bacteria assessed, the antibacterial and antibiofilm activity appear to be weaker in *E. coli*, the Gram-negative bacteria. For the development of antimicrobial medications from *A. paniculata*, and to assess the side effects, experimental research in pre-clinical animal models and clinical trials are required.

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